



Partition efficiencies of newly fabricated universal high-speed counter-current chromatograph for separation of two different types of sugar derivatives with organic–aqueous two-phase solvent systems

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ABSTRACT

A new design of universal high-speed counter-current chromatograph (HSCCC) was fabricated in our laboratory. It holds a set of four column holders symmetrically around the rotary frame at a distance of 11.2 cm from the central axis. By engaging the stationary gear on the central axis of the centrifuge to the planetary gears on the column holder shaft through a set of idle gears, two pairs of diagonally located column holders simultaneously rotate about their own axes in the opposite directions: one forward (type-J planetary motion) and the other backward (type-I planetary motion) each synchronously with the revolution. Using the eccentric coil assembly, partition efficiencies produced by these two planetary motions were compared on the separation of two different types of sugar derivatives (4-methylumbelliferyl and 5-bromo-4-chloro-3-indoxyl sugar derivatives) using organic–aqueous two-phase solvent systems composed of *n*-hexane/ethyl acetate/1-butanol/methanol/water and aqueous 0.1 M sodium tetraborate, respectively. With lower phase mobile, better peak resolution was obtained by the type-J forward rotation for both samples probably due to higher retention of the stationary phase. With upper phase mobile, however, similar peak resolutions were obtained between these two planetary motions for both sugar derivatives. The overall results indicate that the present universal HSCCC is useful for counter-current chromatographic separation since each planetary motion has its specific applications: e.g., vortex CCC by the type-I planetary motion and HSCCC by the type-J planetary motion both for separation of various natural and synthetic products.

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1. Introduction

Counter-current chromatography (CCC) has been widely used for preparative separation and purification of natural products, synthetic compounds and bioactive materials [1–4]. By eliminating the solid support matrix, all charged samples can be recovered from the column without loss of target compound. In the past, various types of CCC instruments have been developed. Among them, type-J multilayer coil planet centrifuge (CPC) and cross-axis CPC have proven to be the most effective models for performing CCC separation. In general, type-J multilayer CPC is useful for separation with organic–aqueous two-phase solvent systems and cross-axis CPC with aqueous–aqueous polymer phase systems. In our laboratory, the improved small-scale model of cross-axis CPC has been

developed and applied to separation of stable proteins [5] and enzymes such as several types of ribonucleases [6] and collagenases [7] with aqueous–aqueous polymer phase systems.

It has been reported that type-I CPC is useful for the separation of DNP-amino acids [8] and dipeptides [8–11] with organic–aqueous two-phase solvent systems. In this type-I CPC, the column rotates about its axis synchronously in the opposite direction to the revolution whereas in the type-J CPC, the column rotates about its axis synchronously but in the same direction to the revolution. In the past, comparison of stationary phase volume retention between type-I and type-J planetary motions has been reported using a large universal CPC [12], but the comparison in partition efficiency between these two planetary motions has not been reported yet. In the present study, we newly designed a compact model of universal CPC with a set of 4 column holders on the rotary frame in such a way that it can perform the type-J and the type-I planetary motions each by a pair of diagonally mounted columns. In this paper, the partition efficiency of these

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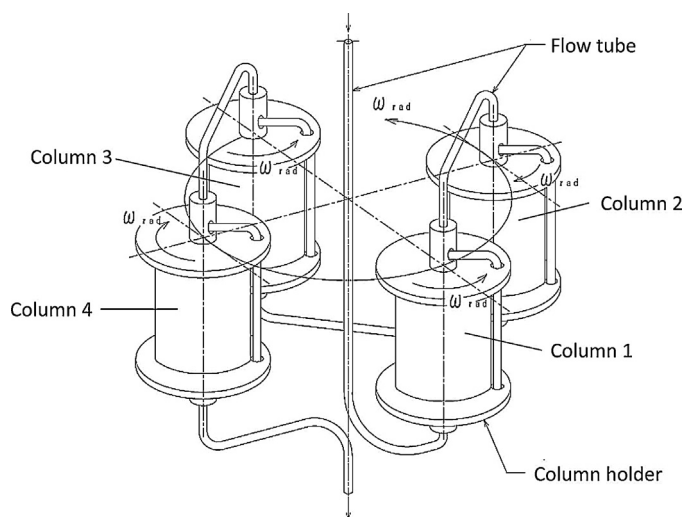


Fig. 1. Schematic illustration of twist-free single-line flow tube distribution for the type-J forward and the type-I backward columns in the universal HSCCC instrument.

two planetary motions is compared by analytical separation of two sets of sugar derivative mixtures with a two-phase solvent system composed of *n*-hexane/ethyl acetate/1-butanol/methanol/water or aqueous 0.1 M sodium tetraborate solution each at a suitable volume ratio.

2. Apparatus

The universal HSCCC employed in the present studies was newly constructed at the Machining Technology Center of Nihon University, Chiba, Japan. The design and fabrication of the apparatus are described below.

2.1. Design of the apparatus

In order to achieve simple and stable planetary motion of the coiled separation column, the CCC apparatus should be designed in such a way that at least a set of two columns of almost the same weight are symmetrically set around the central axis of the centrifuge. The universal HSCCC fabricated for the present study was designed so that four coiled separation columns were uniformly arranged around the central axis as shown in Fig. 1. In this design, one set of diagonally located columns (columns 1 and 3) undergoes the type-J planetary motion and the other set (columns 2 and 4) undergoes the type-I planetary motion. These two planetary motions of the column are achieved by engaging the central stationary sun gear to the identical planetary gear on the holder shaft through the idler gears, one (large gear) for the type-I planetary motion and two (large and small gears) for the type-J planetary motion as shown in Fig. 2. These columns are connected with a single line of flow tubing which is not twisted during the revolution so that the solvent can be continuously eluted through the column without the conventional rotary seal device which often causes leakage and contamination. The present universal HSCCC apparatus simultaneously performs the type-J forward and the type-I backward planetary motions under stable high speed revolution. With this apparatus, it is easy to compare the partition efficiency between two different planetary motions of the type-J forward rotation and the type-I backward rotation, under otherwise identical experimental conditions.

In the present apparatus, the distance from the central revolution axis to the column rotation axis is 11.2 cm, the diameter of



Fig. 2. Photograph of the rotary frame equipped with four column holders in the universal HSCCC instrument.

cylinder shaft of column holder is 3 cm, and the diameter of the flange is 7 cm.

2.2. Preparation of coiled column assembly

For evaluation of partition efficiency on the newly fabricated universal HSCCC, the eccentric coil assembly was used, because the eccentric coil generally produces higher peak resolution and

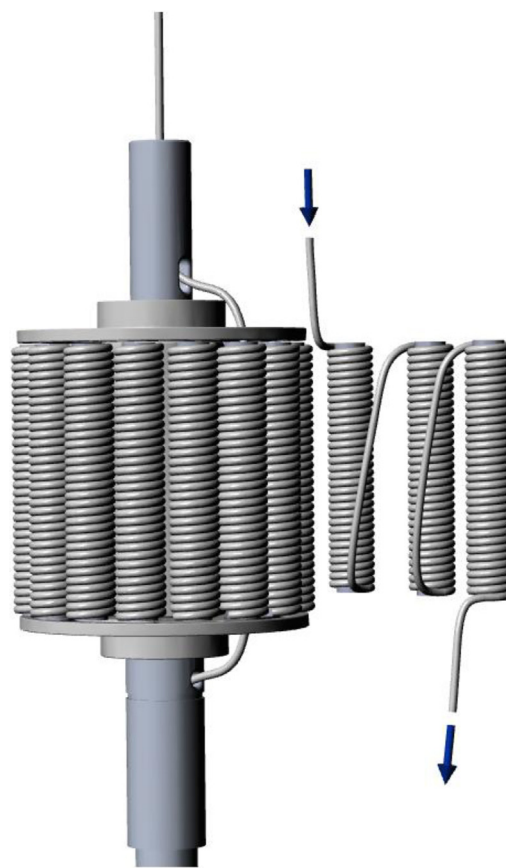


Fig. 3. Schematic drawing of the eccentric coil assembly used in the present study. The arrows show the elution direction when using the lower phase of the two-phase solvent system as the mobile phase.

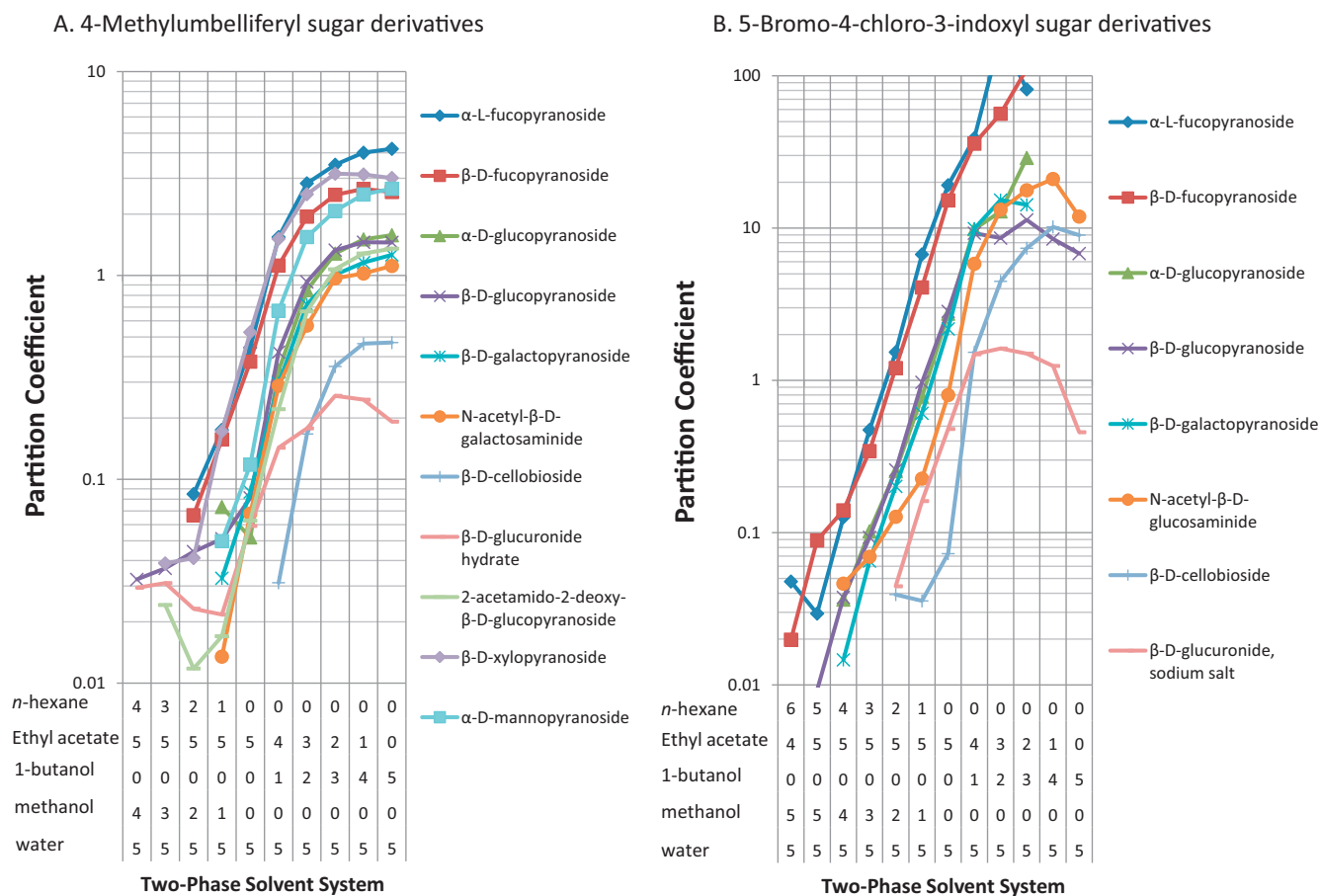


Fig. 4. Measurement of the partition coefficient of two different sugar derivatives using the *n*-hexane/ethyl acetate/1-butanol/methanol/water system.

better stationary phase retention in analytical scale [13,14]. Fig. 3 illustrates the schematic drawing of the eccentric coil assembly. This coil assembly was prepared by winding a 1 mm ID PTFE (polytetrafluoroethylene) tubing (Flon Kogyo, Tokyo, Japan) onto 5 cm long, 5 mm OD nylon pipes making a series of tight right-handed coils. These coil units were arranged symmetrically around the holder hub of 5 cm OD in such a way that the axis of each coil unit is parallel to the holder axis. A pair of assemblies was mounted on the rotary frame on the diagonally located column holders for the desired planetary motion and serially connected with the flow tube (Fig. 1). The total column capacity was 31.5 mL.

2.3. Type-I and type-J planetary motions

Analysis of type-I and type-J planetary motions has been reported earlier [12]. As previously shown in the paper, these two planetary motions show a great difference: type-I planetary motion shows uniformly rotating centrifugal force field at every point on the column. In contrast, the type-J planetary motion produces a complex pattern of the centrifugal force vectors which are different in every point on the holder. Performance of these two planetary motions was also compared by retention of the stationary phase in a single layer of coiled column coaxially wound around the holder ($\beta=0.25$) [12]. The results indicated that the type-J planetary motion can yield a satisfactory level of stationary phase retention except for 1-butanol/water and chloroform-acetic acid-water (2:2:1, v/v/v), where the type-I planetary motion show poor retention of stationary phase in all the solvent systems tested.

3. Experimental

3.1. Reagents

All organic solvents and reagents used for HSCCC separation were of reagent grade. The test samples of sugar derivatives were purchased from three companies as follows:

5-Bromo-4-chloro-3-indoxyl- α -L-fucopyranoside, 5-bromo-4-chloro-3-indoxyl- β -D-fucopyranoside, 5-bromo-4-chloro-3-indoxyl- α -D-glucopyranoside, 5-bromo-4-chloro-3-indoxyl- β -D-glucopyranoside, 5-bromo-4-chloro-3-indoxyl- β -D-galactopyranoside, 5-bromo-4-chloro-3-indoxyl- β -D-cellobioside, 4-methylumbelliferyl- α -L-fucopyranoside, 4-methylumbelliferyl- β -D-fucopyranoside, 4-methylumbelliferyl- α -D-glucopyranoside, 4-methylumbelliferyl- β -D-glucopyranoside, 4-methylumbelliferyl- β -D-galactopyranoside and 4-methylumbelliferyl- β -D-cellobioside were purchased from BIOSYNTH AG (Staad, Switzerland). 4-Methylumbelliferyl-N-acetyl- β -D-galactosaminide, 4-methylumbelliferyl- β -D-glucuronide hydrate and 4-methylumbelliferyl- β -D-xylopyranoside were obtained from Sigma-Aldrich (St. Louis, USA). 5-Bromo-4-chloro-3-indoxyl-N-acetyl- β -D-glucosaminide, 5-bromo-4-chloro-3-indoxyl- β -D-glucuronide, sodium salt, 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside and 4-methylumbelliferyl- α -D-mannopyranoside were obtained from Wako Pure Chemicals (Osaka, Japan).

3.2. Partition coefficient determination

The partition coefficient (K) value of each sugar derivative was measured using the simple test tube method. The organic-aqueous

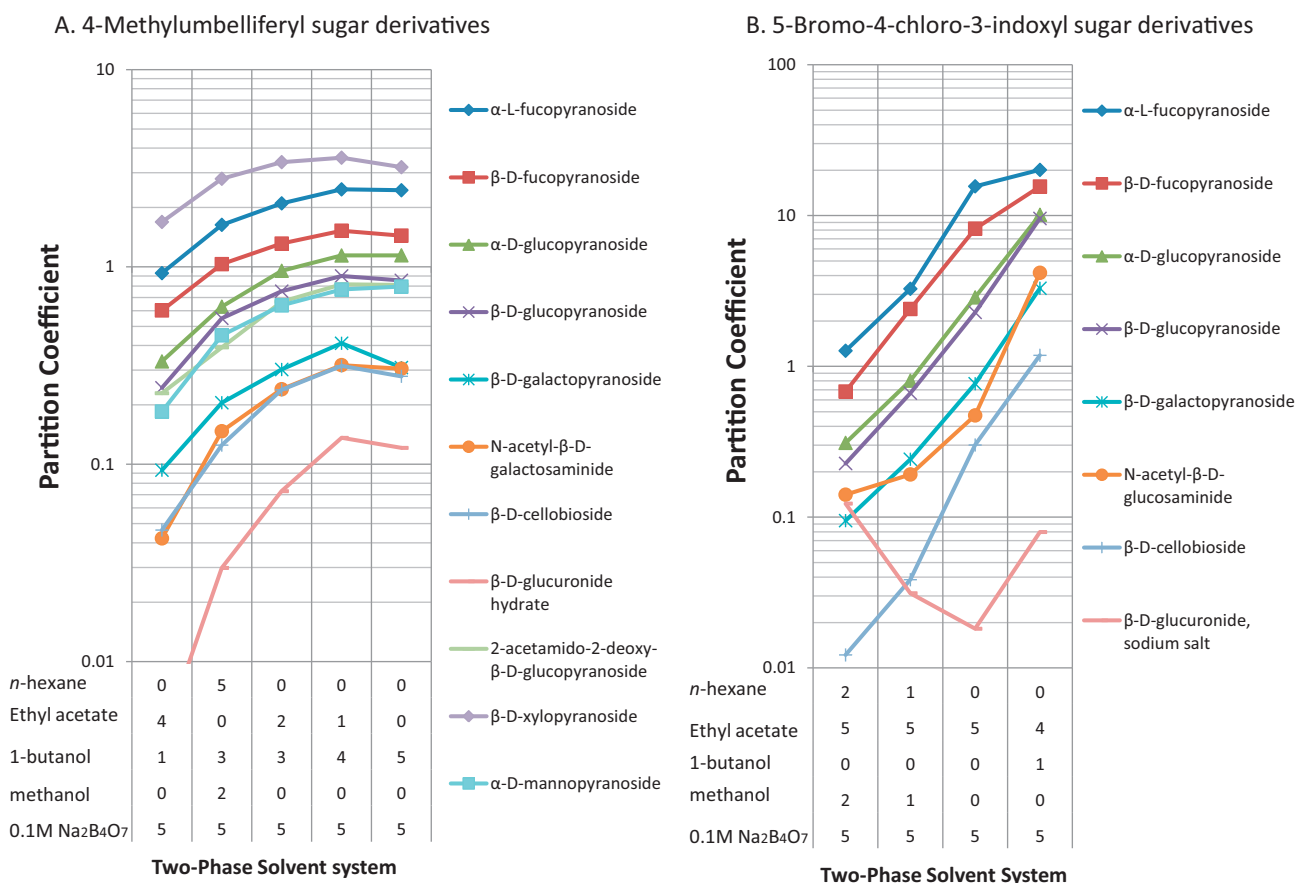


Fig. 5. Measurement of the partition coefficient of two different sugar derivatives using the *n*-hexane/ethyl acetate/1-butanol/methanol/aqueous 0.1 M sodium tetraborate system.

two-phase solvent system examined in the present study was composed of *n*-hexane/ethyl acetate/1-butanol/methanol/water or aqueous 0.1 M sodium tetraborate each at a desired volume ratio according to the method by Oka et al. [15] as follows: two milliliters of each phase of an equilibrated solvent system were delivered into a test tube to which about 1 mg of the sample was added. The contents were thoroughly mixed and allowed to settle at room temperature. After the two clear layers were formed, a 1 mL aliquot of each phase was diluted with 2 mL of methanol, and the absorbance was measured at 318 nm for 4-methylumbelliferyl sugar derivatives and at 293 nm for 5-bromo-4-chloro-3-indoxyl sugar derivatives using a spectrophotometer (Model UV-1600, Shimadzu Corporation, Kyoto, Japan). The *K* value was obtained by dividing the absorbance value of the upper organic phase by that of the lower aqueous phase.

3.3. Preparation of two-phase solvent system and sample solutions

The organic–aqueous two-phase solvent system used in the present study was prepared by mixing water (aqueous 0.1 M sodium tetraborate solution for 5-bromo-4-chloro-3-indoxyl sugar derivatives) and the organic solvent including *n*-hexane, ethyl acetate, 1-butanol and methanol at the desired volume ratio. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, allowing the mixture to settle into two clear layers before use.

The sample solution was prepared by dissolving the standard sample mixture with equal volumes of each phase of the two-phase solvent system used for CCC separation.

3.4. CCC separation of two different sugar derivatives

For each separation, the coil was first filled with the stationary phase followed by injection of the sample solution (ca. 1 mL) into the column inlet with a syringe. Then, the mobile phase was pumped into the column at a flow rate of 0.3 mL/min in the head to tail elution mode using a reciprocating pump (Model KHU-W-52, Kyowa Seimitsu, Co., Tokyo, Japan) while the column was rotated at 1000 rpm. The effluent from the outlet of the column was collected into test tubes at 0.6 mL/tube using a fraction collector (Model CHF100AA, Advantec, Tokyo, Japan).

3.5. Analysis of CCC fractions

Each fraction of sugar derivatives was diluted with 2 mL of methanol and the absorbance was measured at 318 nm for 4-methylumbelliferyl sugar derivatives and at 293 nm for 5-bromo-4-chloro-3-indoxyl sugar derivatives.

4. Results and discussion

4.1. Selection of suitable two-phase solvent systems for CCC separation

The partition efficiency of the universal HSCCC was examined using two different types of sugar derivatives, 4-methylumbelliferyl sugar derivatives and 5-bromo-4-chloro-3-indoxyl sugar derivatives, which have hydrophilic sugar moiety and hydrophobic functional moiety, respectively, in each molecule. In order to achieve efficient CCC separations, a suitable two-phase

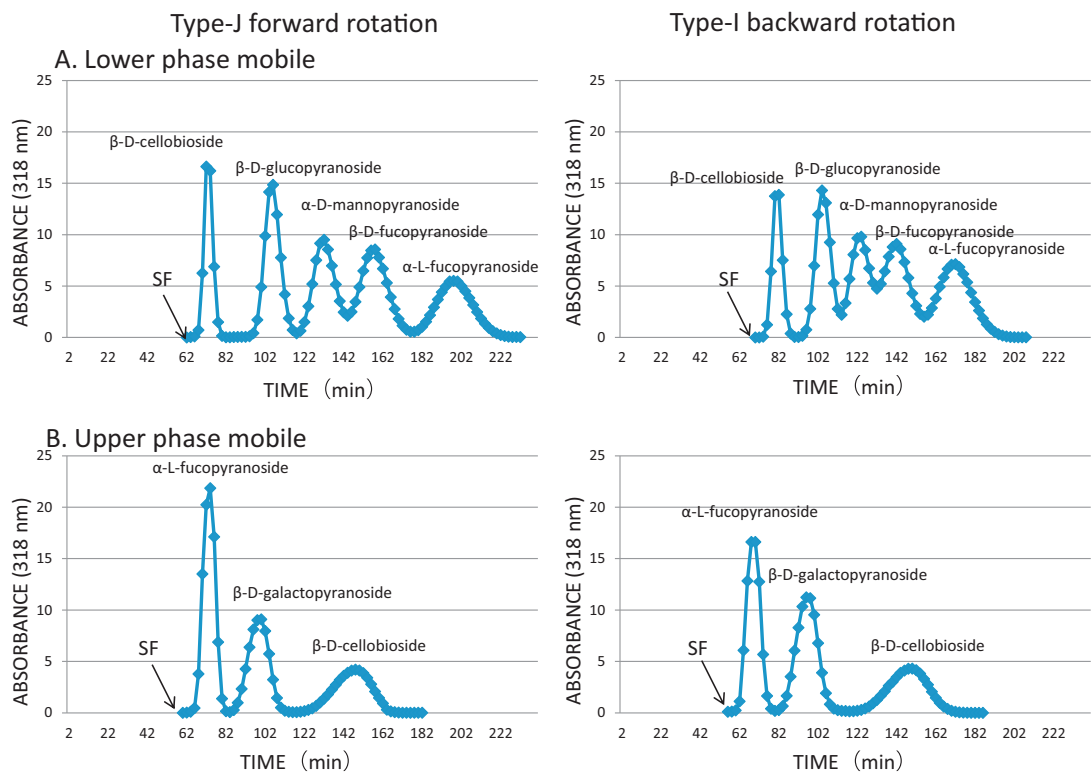


Fig. 6. CCC separation of 4-methylumbelliferyl sugar derivatives using the universal HSCCC with the type-J forward rotation and the type-I backward rotation. Experimental conditions: apparatus: the universal HSCCC equipped with eccentric coil assemblies, 1.0 mm I.D. × 2.0 mm O.D., right-handed coils, 31.5 mL capacity; sample: 4-methylumbelliferyl derivative of β-D-cellobioside (1 mg), β-D-glucopyranoside (1 mg), α-D-mannopyranoside (1 mg), β-D-fucopyranoside (1 mg) and α-L-fucopyranoside (1 mg) for lower phase mobile, and α-L-fucopyranoside (1 mg), β-D-galactopyranoside (1 mg) and β-D-cellobioside (1 mg) for upper phase mobile; solvent system: *n*-hexane/ethyl acetate/1-butanol/methanol/water (0:3:2:0:5) for lower phase mobile and (0:1:4:0:5) for upper phase mobile; rotation and revolution speed: 1000 rpm; rotation and revolution direction: (A) lower phase mobile (outward elution: the lower mobile phase flows from inside of upper part to outside of lower part of the column): type-J forward rotation: rotation: clockwise (CW) (the direction defines from the view of the upper side of the apparatus), revolution: clockwise (CW), type-I backward rotation: rotation: CW, revolution: counterclockwise (CCW), (B) upper phase mobile (inward elution: the upper mobile phase flows from outside of lower part to inside of upper part of the column): type-J forward rotation: rotation: CCW, revolution: CCW, type-I backward rotation: rotation: CCW, revolution: CW; flow rate: 0.3 mL/min; fractionate: 0.6 mL/tube, detection: 318 nm; SF = solvent front.

solvent system should be selected for each sample according to their hydrophobicity. The partition coefficient (*K*) value of each sugar derivative was measured using the simple test tube method. The organic–aqueous two-phase solvent system examined in the present study was composed of *n*-hexane/ethyl acetate/1-butanol/methanol/water at a desired composition ratio according to the method by Oka et al. [15]. Fig. 4A illustrates the change of *K* values of 4-methylumbelliferyl sugar derivatives at different volume ratios of the above solvent system. As the hydrophobicity of the two-phase solvent system is decreased,

the *K* value of each sugar derivative was increased. When using 0.1 M sodium tetraborate solution instead of water to make an anionic complex, the *K* value of each sugar derivative was increased almost in parallel as the hydrophobicity of the solvent system was decreased (Fig. 5A). The *K* value of each sugar of 5-bromo-4-chloro-3-indoxyl derivative was also measured using the *n*-hexane/ethyl acetate/1-butanol/methanol/water system. As illustrated in Fig. 4B, reduced hydrophobicity of the solvent system produced higher *K* values in these sugar derivatives than those of 4-methylumbelliferyl sugar derivatives. By adding sodium

Table 1
Analytical values obtained from the universal HSCCC separation of 4-methylumbelliferyl and 5-bromo-4-chloro-3-indoxyl sugar derivatives.

Rotation	Mobile phase	Resolution (Rs)				Stationary phase retention (%)
4-Methylumbelliferyl sugar derivative						
Type-J	LP	Cel/Glc	Glc/Man	Man/β-D-Fuc	β-D-Fuc/α-L-Fuc	43.6
Type-I	LP	2.38	1.25	0.96	1.19	31.8
		1.70	0.91	0.69	0.88	
		α-L-Fuc/Gal	Gal/Cel			
Type-J	UP	1.52	1.63			41.0
Type-I	UP	1.50	1.65			41.9
5-Bromo-4-chloro-3-indoxyl sugar derivative						
		Cel/Glc	Glc/β-D-Fuc			
Type-J	LP	1.66	2.32			38.7
Type-I	LP	1.10	1.60			28.3
		α-L-Fuc/Glc	Glc/Gal			
Type-J	UP	1.04	1.58			29.5
Type-I	UP	1.07	1.63			27.4

Abbreviations: LP: lower phase; UP: upper phase.

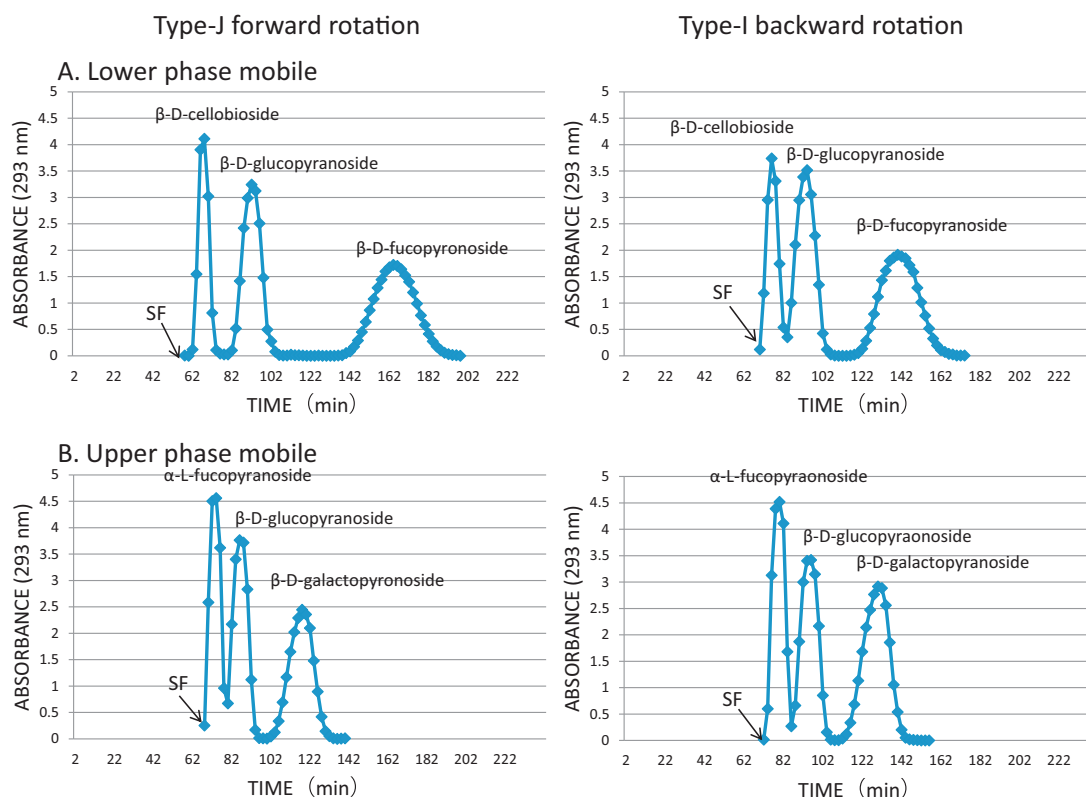


Fig. 7. CCC separation of 5-bromo-4-chloro-3-indoxyl sugar derivatives using the universal HSCCC with the type-J forward rotation and type-I backward rotation. Experimental conditions: sample: 5-bromo-4-chloro-3-indoxyl derivative of β -D-cellobioside (1 mg), β -D-glucopyranoside (1 mg) and β -D-fucopyranoside (1 mg) for lower phase mobile, and α -L-fucopyranoside (1 mg), β -D-glucopyranoside (1 mg), and β -D-galactopyranoside (1 mg) for upper phase mobile; solvent system: *n*-hexane/ethyl acetate/1-butanol/methanol/aqueous 0.1 M sodium tetraborate solution (1:5:0:1:5) for lower phase mobile and (0:5:0:0:5) for upper phase mobile, detection: 293 nm. Other experimental conditions are same as those described in Fig. 6. SF = solvent front.

tetraborate in water at the concentration of 0.1 M, the interval of *K* value was slightly spread between the volume ratios of (2:5:0:2:5) and (0:4:1:0:5) (Fig. 5B). After a series of experiments the solvent system for 4-methylumbelliferyl sugar derivatives was selected as *n*-hexane/ethyl acetate/1-butanol/methanol/water (0:3:2:0:5) with lower phase mobile and (0:1:4:0:5) with upper phase mobile, and for 5-bromo-4-chloro-3-indoxyl sugar derivatives, *n*-hexane/ethyl acetate/1-butanol/methanol/aqueous 0.1 M sodium tetraborate solution (1:5:0:1:5) with lower phase mobile and (0:5:0:0:5) with upper phase mobile.

4.2. CCC separation of two different sugar derivatives at type-J forward and type-I backward rotations

The choice of the eccentric helical column was made according to the following reasons: in the past the coaxial helical column has been examined in their retention of the stationary phase in both type-I and type-J planetary motions [12]. The results clearly indicated that the type-I planetary motion failed to retain the satisfactory amount of stationary phase in the column. While the eccentric helical column has been successfully used in the type-I CCC system [8], it has not been used for the type-J CCC system yet. Therefore, it is interesting to compare the performance of both types of the HSCCC system using the eccentric helical column. Also it should be noted here that in the type-I planetary motion every portion on the column holder is subjected to exactly the same centrifugal force field regardless of its β -value.

Fig. 6A illustrates the CCC separation of five 4-methylumbelliferyl sugar derivatives including β -D-cellobioside (Cel), β -D-glucopyranoside (Glc), α -D-mannopyranoside (Man), β -D-fucopyranoside (β -D-Fuc) and α -L-fucopyranoside (α -L-Fuc)

obtained using a two-phase solvent system composed of *n*-hexane/ethyl acetate/1-butanol/methanol/water (0:3:2:0:5) with lower phase mobile. Five sugar derivatives were well separated from each other at the type-J forward rotation (upper left side), while less efficient separation was obtained at the type-I backward rotation (upper right side) where the third peak of α -D-mannopyranoside and the fourth peak of β -D-fucopyranoside were only partially separated under otherwise identical experimental conditions. Fig. 6B illustrates the CCC separation of 4-methylumbelliferyl sugar derivatives of α -L-fucopyranoside, β -D-galactopyranoside (Gal) and β -D-cellobioside using *n*-hexane/ethyl acetate/1-butanol/methanol/water at the volume ratio (0:1:4:0:5) with upper phase mobile. Although the type-J forward rotation produced better separation of three derivatives than the type-I backward rotation with lower phase mobile, almost similar separation of these compounds was obtained by both methods with upper phase mobile. These results suggest that the difference on peak resolution between these two planetary motions is mainly caused by the difference in the amount of stationary phase retained in the column.

Fig. 7A illustrates the CCC separation of three 5-bromo-4-chloro-3-indoxyl sugar derivatives including β -D-cellobioside, β -D-glucopyranoside and β -D-fucopyranoside obtained using the lower phase of *n*-hexane/ethyl acetate/1-butanol/methanol/aqueous 0.1 M sodium tetraborate solution (1:5:0:1:5) as the mobile phase. Better separation was obtained by the type-J forward rotation (upper left side) than the type-I backward rotation (upper right side), the results of which was similar to those obtained using 4-methylumbelliferyl sugar derivatives with lower phase mobile. Fig. 7B illustrates the CCC separation of 5-bromo-4-chloro-3-indoxyl derivatives of α -L-fucopyranoside, β -D-glucopyranoside

and β -D-galactopyranoside obtained using the upper phase of *n*-hexane/ethyl acetate/1-butanol/methanol/aqueous 0.1 M sodium tetraborate solution (0:5:0:0:5) as the mobile phase. Almost similar separation was obtained between the type-J forward rotation (lower left side) and the type-I backward rotation (lower right side).

Table 1 summarizes the analytical values calculated from the chromatograms described above (Figs. 6 and 7). With lower phase mobile, better stationary phase retention was obtained by the type-J forward rotation than by the type-I backward rotation in both separations of 4-methylumbelliferyl and 5-bromo-4-chloro-3-indoxyl sugar derivatives. The overall experimental results indicated that, when the lower phase is used as the mobile phase, better peak resolution is achieved in the type-J forward rotation than in the type-I backward rotation under otherwise identical experimental conditions, whereas almost similar resolution was obtained between the type-J forward rotation and the type-I backward rotation with upper phase mobile.

5. Conclusions

The universal HSCCC instrument was newly designed and fabricated in our laboratory. The apparatus performs two different planetary motions, the type-J forward rotation and the type-I backward rotation. Using an eccentric separation column, performance of these two planetary motions was examined in the separation of two sets of sugar derivative test samples each with a suitable two-phase solvent system. When the lower phase was used as the mobile phase, better peak resolution was obtained by the type-J forward rotation than the type-I backward rotation under otherwise identical experimental conditions, however, when the upper phase was used as the mobile phase, almost similar separations were obtained between these two planetary motions. These results suggest that the retention of the stationary phase plays an important

role in the CCC separation. The present apparatus may provide useful applications for two different types of CCC separations: the type-I rotation for vortex CCC [16–18] and type-J rotation for HSCCC, both for the separation of natural and synthetic products.

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